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Evidence of circulation of an epidemic strain of *Francisella tularensis* in France by Multi Spacer Typing

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ABSTRACT

Multispacer typing (MST) was used to type 10 *Francisella tularensis* strains detected in French patients. Incorporating 79 Swedish *F. tularensis* strains, phylogenetic analysis demonstrated that that in spite tularemia appears as a sporadic disease in France, it is caused by an epidemic cluster of strains.

Francisella tularensis, the causative agent of tularemia in mammalian species, is a facultative intracellular bacterial species. Four subspecies of *F. tularensis*, subsp. *tularensis*, subsp. *holarctica*, subsp.*novicida*, and subsp. *mediasiatica*, have been identified and each of them has distinct virulence and geographic distribution (1). Human tularemia is endemic in certain areas of the northern hemisphere and may be present in various clinical forms depending on the route of inoculation and the virulence of the *F. tularensis* strains involved (2). The less frequent but more severe clinical form, respiratory tularemia (type A tularemia), is caused by inhalation of highly virulent *F. tularensis* subsp. *tularensis* and has mainly been identified in Northern America (2). The most common clinical manifestation of *F. tularensis* infection, type B tularemia, is caused by *F. tularensis* subsp. *holarctica.* It is usually contracted from the bite of an arthropod vector that previously fed on an infected animal or contact with infected animals. Type B tularemia is characterized by the development of a local painful lesion at the site of inoculation, tender regional lymphadenopathy, fever and other constitutional symptoms including malaise, headache, anorexia, and myalgia (3). Type B tularemia is endemic to countries of the Northern Hemisphere and is rarely life threatening but may lead to long-lasting complications (e.g., lymph node suppuration). In France, tularemia is a rare and mostly sporadic disease and only type B tularemia has been reported (4). Several animal outbreaks have been recorded (5). In 2004, an outbreak of airborne tularemia caused by *F. tularensis* subsp. *holarctica* occured in western France, which suggested a transmission from dog to humans by inhalation of contaminated particles from the dog's fur (6).

While each subspecies of *F. tularensis* has distinct virulence and geographic distribution, the genetic difference among them is minor, making individual discrimination of *F. tularensis* strains and epidemiological surveillance of tularemia extremely difficult (7). Multispacer typing

(MST) incorporating four highly variable intergenic spacers which were selected by genome comparison exhibited more powerful capacity of individual discrimination of *F. tularensis* strains and more reasonable subspecies classification than multilocus variable number tandem repeat analysis (MLVA) (8). In this study, MST was tested to direct typing of *F. tularensis* strains detected in human specimens in France.

This study was performed after agreement of the ethical committee of IFR 48 under 08-014. Ten French patients with swollen lymph nodes and with diagnosis of type B tularemia on the basis of clinical manifestations and serological test in 2008 were included in this study. Lymph node biopsies from these patients were frozen at -20°C then referred to our laboratory.

Total genomic DNA was extracted from the lymph nodes using QIAamp Tissue kit (QIAGEN, Hilden, Germany) according to the manufacturer's recommendations. Specific PCR detection of *F. tularensis* on the DNA extracts was performed using a real-time quantitative PCR assays targeting the 17kDa major membrane protein precursor of the bacterium. This PCR included the primers Tul4F (ATTACAATGGCAGGCTCCAGA) and Tul4R (TGCCCAAGTTTTATCGTTCTTCT) and a Taqman probe (Tul4, 6-FAM-TTCTAAGTGCCATGATACAAGCTTCCCAATTACTAA-TAMRA). PCR was done in a LightCycler instrument (Roche Biochemicals, Mannheim, Germany).The PCR mixture included a final volume of 20µl with 10µl of the Probe Master kit (Qiagen), 0.5µl (10pmol/µl) of each primer, 5µl (2µmol/µl) of probe, 3µl of distilled water, and 2µl of extracted DNA. The amplification conditions were as follows: an initial denaturation step at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C, annealing and elongation at 60°C for 60s, with fluorescence acquisition in single mode. After every five patients samples, negative controls (sterile distilled water, PCR mix, and biopsy samples from healthy humans) were inserted. MST

incorporating 4 highly variable intergenic spacers, S1, S2, S3, and S4, which were selected by genome comparison was performed as previously described (9). Briefly, these 4 highly variable intergenic spacers were amplified and sequenced from the genomic DNA of each lymph node biopsy. The sequence variations of concatenation of these four intergenic spacers were then used for discrimination of *F. tularensis* strains detected in these lymph node biopsies. A MST genotype is defined as unique sequence variation of concatenation of these four intergenic spacers. Phylogenetic organization of the 10 French strains in this study and the 79 strains collected in Sweden and one strain collected in France in previous study was inferred on the basis of concatenation of these 4 intergenic spacer sequences using the UPGMA, Neighbor-Joining and Maximum Parsimony methods within the MEGA 4.1 software. We added to the present analysis the 4 intergenic spacer sequences of *F. tularensis* subsp. *tularensis* strain Schu 4, *F. tularensis* subsp.*novicida* strain GA99-3549, and *F. tularensis* subsp. *mediasiatica* strain FSC147 with GenBank accession numbers, NC_006570, NZ_AAYF00000000, and NC_010677, respectively.

MST, a DNA sequence-based genotyping method using highly variable intergenic spacers as typing markers which are selected on the basis of genome comparison, is a byproduct of genome biology in the genomic era (10). Since 2004, MST has been applied to several important human pathogenic bacteria and exhibited great power for strain typing. In our previous study, MST represented by four highly variable spacers showed good discriminatory power and gave a consistent phylogenetic classification of the studied strains (9). Using this technique, we identified 4 new genotypes of spacer S1, types 19-22, but no new genotype of spacers S2, S3, and S4 (Figure 1). Newly identified genotypes of each spacer identified in the present study were deposited in GenBank (accession number: EU877932- EU877935). In total, 4 new MST

genotypes were identified among the 10 French strains (Figure 1). The sequence identity of concatenation of 4 intergenic spacers of 11 French strains is higher as compared to that of 79 Swedish strains with 97.39% versus 88.62% respectively (Table 1). Phylogenetic analysis based on different methods exhibits similar organization. The 31 MST genotypes identified in French and Sweden strains of subsp. *holarctica* were grouped into 3 clusters and *F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. *mediasiatica*, and *F. tularensis* subsp. *novicida* formed outgroups, respectively (Figure 1). Interestingly, the eleven French strains grouped tightly into one cluster and all the 79 Sweden strains were grouped into 2 other Swedish clusters, respectively (Figure 1).

In our analysis of *F. tularensis* subsp. *holarctica*, we found that one cluster is unique to French strains which exhibit relatively lower genetic diversity than previously typed Swedish strains (Table 1 and Figure 1). This confirms that French cases of tularemia that appear as rare and sporadic, is in fact an epidemic due to closely related strains as previously observed in central and western Europe (11). In France, tularemia is a notifiable disease. Every year, between 20 and 40 cases are reported (http://invs.sante.fr/surveillance/tularemie/default.htm). Direct diagnosis is done in approximately 20% of cases, mostly by PCR but only 1-3 *F. tularensis* strains are isolated yearly due to the fastidious cultural properties of *F. tularensis*. The difficulty of isolation and culture of *F. tularensis* from human specimens further hinders epidemiological surveillance because many molecular typing approaches are based on cultivated strains (10). Dempsey *et. al* identified a 1.59kb genomic deletion specific to *F. tularensis* subsp. *holarctica* isolates from France and Spain and classified these isolates into an emerging subclone in France and Iberian Peninsula (12). The same picture was observed for strains from Switzerland and France, countries where the epidemic clone was present before the tularemia

outbreak of 1997-1998 in the Iberian Peninsula (11). Our study further supports that the *F. tularensis* subsp. *holarctica* strains in France are an emerging subclone. Identification of several subclusters among *F. tularensis* subsp. *holarctica* also confirms that it is a dynamic subspecies rather than a homogeneous group as observed in *F. tularensis* subsp. *tularensis* in which two subpopulations were identified (13). The non homogeneous structure of *F. tularensis* subsp. *holarctica* population was also recently observed in Japan by using MLVA and in a global study using microarray SNP genotyping (14;15). This study confirms the usefulness of MST in epidemiological study of *F. tularensis* infection in France and even in Europe where tularemia is mainly caused by subsp *holarctica*, a particularly conserved subspecies (7). To obtain a clearer population structure of *F. tularensis* subsp. *holarctica*, MST, that requires performing four short sequences for powerful discrimination, may be useful in the future large scale epidemiologic studies incorporating a large number of strains.

Table 1. Comparison of genetic diversity of French and Swedish strains of *F. tularensis* subsp. *holarctica* by DNA sequence variation, number of MST genotype, and number of cluster based on four highly variable intergenic spacers.

***F. tularensis* subsp. *holarctica* strains**	**Strain number***	**Number of MST genotype***	**Number of cluster***	**Sequence identity****	**Sequence difference****
French strains	11	5	1	97.39%	2.61%
Swedish strains	79	26	2	88.62%	11.38%

* also see Figure 1

** The DNA sequence identity and difference of concatenation of 4 intergenic spacers were generated by sequence alignment using Clustal W program (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_clustalwan.html).

Figure 1. Dendrogram showing the phylogenetic organization of the studied 10 French strains of *F. tularensis* subsp *holarctica* with 80 strains of *F. tularensis* subsp *holarctica* from previous study (9) and *F. tularensis* subsp. *tularensis* strain Schu 4, *F. tularensis* subsp. *mediasiatica* strain GA99-3549, and *F. tularensis* subsp.*novicida* strain FSC147 which have genome sequences in GenBank. Sequences from the four intergenic spacers were concatenated.

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